Strategies for Assessing the Implications of Malformed Frogs for Environmental Health

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The recent increase in the incidence of deformities among natural frog populations has raised concern about the state of the environment and the possible impact of unidentified causative agents on the health of wildlife and human populations. An open workshop on Strategies for Assessing the Implications of Malformed Frogs for Environmental Health was convened on 4–5 December 1997 at the National Institute of Environmental Health Sciences in Research Triangle Park, North Carolina. The purpose of the workshop was to share information among a multidisciplinary group with scientific interest and responsibility for human and environmental health at the federal and state level. Discussions highlighted possible causes and recent findings directly related to frog deformities and provided insight into problems and strategies applicable to continuing investigation in several areas. Possible causes of the deformities were evaluated in terms of diagnostics performed on field amphibians, biologic mechanisms that can lead to the types of malformations observed, and parallel laboratory and field studies. Hydrogeochemistry must be more integrated into environmental toxicology because of the pivotal role of the aquatic environment and the importance of fates and transport relative to any potential exposure. There is no indication of whether there may be a human health factor associated with the deformities. However, the possibility that causal agents may be waterborne indicates a need to identify the relevant factors and establish the relationship between environmental and human health in terms of hazard assessment. *Key words*: amphibian malformation, environmental health. *Environ Health Perspect* 108:83–90 (2000). [Online 16 December 1999]
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Over the past two decades there has been a rapid decline in amphibian populations worldwide (*1,2*). Many contributing factors associated with particular regions and species have been identified or speculated and it is likely that there are a variety of important environmental interactions that can enhance or ameliorate detrimental effects on local amphibian populations. Over the last 3 years considerable attention has been given to observations of apparent increases in morphologic abnormalities among several amphibian species such as the northern leopard frog (*Rana pipiens*), green frogs (*Rana clamitans*), bull frog (*Rana catesbeiana*), and mink frog (*Rana septentrionalis*). Reports of abnormal amphibians in North America have come from regions of Canada and from several U.S. states including Minnesota, Wisconsin, and Vermont. It is not clear how the abnormalities relate to amphibian population decline or what the more far-reaching implications for environmental decay may be. However, because amphibians may be relevant natural sentinels of environmental contaminants and the importance of aquatic systems to human and ecologic health, there is ample justification to investigate the potential causes for the abnormalities (*3*).

Supernumerary hindlimbs and a few cases of missing limbs among amphibians have been documented (*4–6*). These accounts have usually involved specific species and sites that later were found to be normal. However, the recent increases in the frequencies of malformations involve several species and widespread sites as well as a greater incidence of ectromelia (missing limbs) and ectrodactyly (missing digits) (*7*). Deformities can be generated in amphibians by several means, including changes in predation, endoparasite infestation and disease, ultraviolet (UV) radiation (direct or by chemical modification), mineral depletion (e.g., calcium and magnesium), and natural or man-made chemicals (*8–16*). Certain retinoids are capable of producing many types of developmental abnormalities in a range of species with differences in response within species determined primarily by dose and by time of exposure (*17–21*). There is also ample evidence to support complex interaction among endocrine factors (*22–24*).

The current widespread deformities among frogs seem to suggest a recent environmental change and several issues have been raised.

- What are the relative increases in malformation above the true historical background? There has been some discussion that a proportion of the current observations is due to heightened awareness among the observers.
- Is there an association between increased incidence of malformations and types with specific sites or conditions? Accurate field studies of natural populations are difficult to conduct and are subject to significant sampling variation.
- Can environmental agents or conditions that are capable of causing abnormalities in model systems be identified in the field? This issue is further complicated by local environmental matrices, which may greatly alter the effect of any single agent.
- What are the possibilities for detrimental effects on the health of other natural species, ecosystems, and humans?

A major consideration in the issue of frog deformity is our dependence on water quality. Therefore, several groups from academic institutions and agencies at the state and federal level have expressed an interest in the status of the investigations. However, the frog deformity issue is somewhat unique

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within the context of the normal conduct of scientific investigation. Many of the initial observations are anecdotal and the current set of cause-and-effect hypotheses has not yet been sufficiently tested and reported in the peer-reviewed literature. Concurrently, research design decisions must be made for the rapidly approaching next cycle of seasonal reproduction and development. The objectives of the Strategies for Assessing the Implications of Malformed Frogs for Environmental Health workshop were to disseminate some of the unpublished frog deformity information in a discussion of possible causes and strategies for assessing the implications of malformed frogs for environmental health. Because of the importance of surface–groundwater flux, water age, and fates related to agents in the aquatic environment, there should also be renewed emphasis on hydrogeochemistry. Finally, there is a need for integration of environmental and human health studies in terms of hazard identification and assessment.

A Perspective on Issues

The investigations into causes for frog malformation have been unusually speculative, highly visible, and at times contentious. However, the progress of scientific understanding can always be improved by focusing less attention on competing speculations and by adhering to the scientific method of hypothesis testing. In the case of deformed amphibians, we also need to include a range of disciplines/expertises in what has evolved into a complex investigation with many interrelated facets. From a field science point of view, greater attention must be given to the reality that laboratory model systems may be selected less for their appropriateness than for their availability and ease of acquisition. Each species has its own evolutionary history and confidence in the direct correlation of surrogate data may be inversely proportional to evolutionary distance. It is also necessary to understand whether the observed effects of concern in the natural populations actually exist or simply result from anecdotal reporting bias. The involvement of experienced field scientists should be an early and integral component in any process designed to understand potential environmental impact. Effects observed in a particular species may indicate environmental deterioration for that species, but the extent to which the effects serve as predictive bioindicators for other species or humans may be unclear.

In the predictive integration of ecologic and human health effects, the issues associated with surrogate/bioindicator/sentinel species become pivotal. Experimentation with the preferred species may be impractical or impossible: it has been at the heart of debates on human health hazards and risk assessments for decades. Experimentation continues with the same uncertainties in the current drive to reduce the numbers and phylogenies of species that may be used predictively. The deformed frogs issue is divided into two perspectives: the use of a convenient surrogate species such as *Xenopus* to guide questions of importance to the natural affected populations and the use of an aquatic species to guide investigations relevant to human health. In both cases the validity of association depends on the continued improvement of mechanistic data that may allow surrogate organisms to contribute to the weight of evidence. One of the most important points to be reaffirmed relative to frog deformities is the need for hypothesis-driven research. In the process of investigation the hypotheses may be disproven, but the concepts and the efforts are not discredited.

Geographic Extent and Incidence

An accurate assessment of change within a population depends on a reference for comparison. Significant progress has been made in the establishment of a national reporting mechanism whereby reports of deformities can be submitted, verified, and catalogued according to location, species, and type of malformation. Once organized and verified, this information is currently available on the Internet (*25*). For each report the database contains the location, date of sampling, number of frogs examined, number of malformations observed, and classification of the types of malformations (missing limbs and digits, extra/split limbs, eye abnormalities, jaw abnormalities, and other unclassified abnormalities). The database could be useful in making generalizations about rates and characterizations among species and areas. However, the data are heavily skewed toward malformations and sites with known deformities. This makes it difficult to use the database to determine relative increase above background. Recently, Hoppe (*26*) estimated that the background incidence of malformation in areas where deformed frogs are being reported is probably in the range of 0.5% and similar background rates estimated by Ouellet (*27*) for an area in Canada. These similarities suggest that some of the areas of Minnesota that were classified "unaffected" for the purpose of ongoing comparative studies may, in fact, be only "less affected." An accurate and more random evaluation of the frequency of abnormalities in natural populations is necessary, along with a better field assessment of areas with putative high frequencies of abnormalities.

A Diagnostic Evaluation

Careful study of the abnormalities can provide important clues about the causal mechanisms. Abnormal development that gives rise to malformations can be caused by genetic or environmental factors (teratogens). Environmental factors include radiation, hyperthermia, low oxygen, high carbon dioxide, poor nutrition, and endogenous or exogenous chemicals. The same teratogen presented at different developmental ages can initiate different malformations and different teratogens presented at the same developmental age can result in similar errors in development and cause the same malformation.

Malformation was the most frequent finding in abnormal newly metamorphosed frogs submitted to the National Wildlife Health Center (Madison, WI) from the Vermont and Minnesota studies during the summer of 1997. The types of malformations reported included completely missing limbs, sometimes with agenesis of the ipsilateral half of the pelvis; multiple limbs sometimes associated with multiple pelvic girdles; abnormal differentiation and growth of the limb (phocomelia); webbing of skin between the rear limb and the body; and abnormal development of craniofacial structures. The data suggest that specific types of malformations seem more prevalent at certain sites and that some of the malformations are associated with poor growth and body condition.

In the limited parasitology work performed on submissions, there did not appear to be a correlation between malformations and the presence of larval flukes (metacercaria), as suggested by some researchers. No association could be found between bacteria isolated from frogs and malformations. Iridovirus was isolated from frogs from only one site in Minnesota and this site had a high prevalence of skin webbing; however, it is not known if the virus is associated causally with skin webbing formation. As more data are gathered from the field regarding the distribution and frequency of abnormalities in newly metamorphosed frogs, we must remember that these data reflect survivors with malformations that were not fatal. This may limit our understanding of events because the portion of frog populations that may have had earlier stage lethal malformations are not being considered.

Biologic Influences of UV Irradiation

Investigators at the U.S. Environmental Protection Agency (EPA) laboratory in Duluth, Minnesota, explored the possible interaction between the insecticide methoprene and UV irradiation. The data generally indicated that methoprene induced lethal developmental anomalies only at very high concentrations (*28*). The effects were not similar to those observed in the field and there were no effects at lower concentrations closer to possible environmental exposure levels. In these experiments the toxicity of methoprene was not increased by simultaneous UV exposure. However, when *Rana pipiens* tadpoles were exposed to UV irradiation for 10 hr/day for > 24 days there was an induction of rear limb and digit deficiencies and approximately 47% of the frogs held under UV light through metamorphosis had limb malformations. These malformations were mostly bilateral and in some cases symmetrical. The results suggest that it may be possible for direct UV irradiation to contribute to limb malformation in *Rana pipiens*, which is one of the species affected in the wild.

Developmental and Molecular Mechanisms

The limb phenotypes can generally be categorized into hypomorphic limbs (missing various skeletal elements), entirely missing limbs, limbs with extra skeletal elements, and entire extra limbs. For each of these phenotypes three general mechanisms (grafting/wounding, UV irradiation, and chemical/molecular) can be evaluated to see whether they could cause the limb abnormalities observed in frogs in the field.

Grafting/wounding. Many experiments have demonstrated that by relocating groups of cells from one position to another within developing or regenerating limbs, it is possible to induce both extra limb structures and missing limb structures. Such tissue arrangements do not cause either entire extra limbs or entirely missing limbs. Although theoretically possible, there is no evidence that the tissue rearrangement mechanism would cause limb abnormalities in frogs in the field. Because frog limbs only regenerate at the early limb bud stages, tissue damage/wounding would not result in a regenerative response in animals that had developed beyond such early stages. Therefore, injuries to limbs in later stage larvae and juvenile frogs could only result in missing distal structures, but not in extra structures. Evidence of traumatic injuries generally has not been seen, and if these injuries were sustained they would need to occur when the limb bud was very small but not kill or mortally wound the tadpole. One report suggested that the implantation of microbeads as a mimic of parasite cysts can result in some limb abnormalities (*8*). However, these abnormalities were restricted to only distal elements in experimental limbs and one control limb, and no extra proximal elements or extra legs were induced. Because it is based on mechanistic and diagnostic data, the physical rearrangement of limb tissues is unlikely to be a significant general mechanism for causing abnormal limb patterns outside of the controlled laboratory environment.

UV irradiation. Recently, as a result of efforts at the U.S. EPA laboratory in Duluth, we have begun to consider UV irradiation a possible mechanism for causing missing limb structures. Based on the anatomy of the experimental frog images, it is clear that UV exposure can cause truncated limbs. Such limbs exhibit a relatively normal pattern along the proximal–distal axis to the level of the truncation, at which point further outgrowth and pattern formation ceases. This phenotype is characteristic of experiments in which the apical epidermis is removed. This regionalized epidermis functions to support limb outgrowth, and when absent or functionally inhibited, outgrowth and pattern formation cease. The apical epidermis is a hypothetical target for UV irradiation in the production of abnormal limbs. It seems unlikely that such a mechanism would give rise to extra limb structures, and there is no evidence that it would cause entirely missing limbs. Direct comparison of the laboratory UV irradiation-induced abnormalities with the hypomorphic limbs on the deformed mink frogs from Minnesota indicates that UV irradiation may be established as a causal agent for hypomorphic limbs in some field situations; however, other agent(s) not related to the UV effect may also be responsible for the hypomorphic limbs examined to date.

Chemical and molecular mechanisms. Chemicals and molecules are intrinsic to the development of an organism and there is abundant evidence that chemicals and molecules can effect limb formation in ways that can account for all of the categories of limb abnormalities. If there is a man-made or naturally occurring chemical in the environment that is causing frog limb deformities, its mode of action is to alter or mimic the function of an intrinsic chemical that is involved in the control of normal development. There are multiple mechanisms that might result in partially missing limbs or additional limb structures. In contrast, entirely missing limbs and entire extra limbs can only be accounted for by a chemical and molecular mechanism. These types of abnormalities may be particularly informative and useful in testing the causal mechanism(s) involved in the extra leg phenotype.

When considering what is known about endogenous molecules that can induce limb abnormalities, it is obvious that the retinoids can induce all limb phenotypes observed in the frogs in the field. This simple observation leads to the strong inference that the agent(s) in the environment is in some way targeting a developmental signaling pathway that is retinoid responsive. There need not be a single agent, and it need not itself be a retinoid. Several molecular pathways are involved in limb pattern formation, and there is evidence for interactions between these pathways such that alterations in one would cause changes in others. It is also likely that there are as-yet unknown pathways as well as unidentified interactions. From a molecular and developmental biology perspective, the multiple-leg phenotype reveals the existence of a currently unidentified developmental pathway specific for the establishment of the hindlimb field. Thus, further basic research on the mechanisms controlling growth and pattern formation in limbs is needed to provide the critical clues for identifying the agent(s) and mechanism involved in the deformed frog syndrome.

Research in Minnesota

The first anecdotal evidence for a possible increase in the incidence of abnormal frogs in Minnesota began to appear in 1994 and 1995. By 1996 the Minnesota Pollution Control Agency (MPCA) in St. Paul, Minnesota, had received reports from over 100 locations; approximately 20 sites were confirmed by biologists. The abnormalities in six species of frog and toads were primarily missing, reduced, and misshapen rear limbs; a few of the animals had extra limbs or were missing eyes. In 1997 the MPCA and the National Institute of Environmental Health Sciences [(NIEHS); Research Triangle Park, NC] formed a partnership. Their goals were to determine if the original anecdotal evidence for an increased incidence of malformed frogs was scientifically valid, to determine whether causal factors could be identified in the aquatic environments where abnormal frogs had been reported, and to assess whether there was any environmental and human health hazard that might result from agents that can be identified as potentially causative. To accomplish these goals, partnerships and collaborations were established with other federal agencies, state agencies, academic institutions, and contract laboratories. Based on MPCA information from 1995 and 1996, a number of sites were identified and given operational classifications of affected or reference based on the previous incidences of abnormal frogs. The paired sites were sampled for water matrix condition (i.e., pH alkalinity, hardness, conductivity, dissolved oxygen, etc.), water and sediment chemistry (targeted metals and analytes such as herbicides and pesticides as well as a full scan for unknowns), and abnormalities among frogs. Biologic assays such as the 4-day frog embryotoxicity assay *Xenopus* [(FETAX); which uses embryos from the South African clawed frog *Xenopus laevis*] and *in vitro* retinoid receptor binding were also conducted.

Studies of frog abnormalities in the paired sites and comparisons with historical data from Minnesota indicate that the frequencies of malformation among wild amphibians have increased dramatically at some locations over the last 3–5 years. Several species of frogs are affected. Some sites have incidences of malformed frogs significantly above reference sites but, given a normal population value in the range of 0.5%, there may not be any true unimpacted sites in the paired Minnesota study.

Data from wild frogs and from FETAX indicate the involvement of waterborne agents (*29*). When combined with the diagnostic evaluation presented here, data from the field and laboratory studies also point to different agents or modifications of a single agent among sites. The types of abnormalities vary and are site specific. There is also significant mortality and developmental retardation associated with several samples from affected sites. Long-term limb malformations have been induced in *Xenopus* using unconcentrated pond water samples from affected sites.

There are no apparent patterns of metal or chemical contaminants in water that malform frogs. Chemical analysis by several groups has failed to demonstrate the presence of methoprene (suggested to be causative because of retinoid-like activity) or its more toxic environmental degradation products in any sites at biologically relevant levels. However, in all cases, agents have been identified in malforming water that are not in nontoxic samples. Teratogenic fractions have been isolated from C-18 column eluents of the limited number of pond water samples that have been fractionated to date. Experiments are underway with the fractions and the compounds that have been identified within those fractions (*30*).

Several lines of evidence suggest the involvement of endocrine factors such as thyroid hormone, retinoids, or mimics. In laboratory assays, the teratogenic effects are significantly reduced or eliminated by the addition of thyroxin to the water (*31*). However, efforts using different cloned retinoid receptor binding assays with water and extracts from a number of affected and reference sites have been inconclusive with respect to any direct correlation between a positive receptor assay and malformation of wild and/or laboratory frogs. The mechanism does not necessarily involve retinoids because of the complex interactions of developmental pathway components.

One groundwater source had teratogenic activity and lethality in *Xenopus* and malformations were induced by well water from some locations. The teratogenic potential of the well water can be reduced or removed by activated carbon filtration in the samples that have been examined to date. There is no additional evidence that would support any human health risk without a determination of exactly what in the well water samples caused the positive results. A study to identify those factors is underway.

Vermont Field Studies

In late summer 1996, malformed frogs were reported by the general public to the Vermont Agency of Natural Resources [(VTANR); Waterbury, VT] from 12 sites in five counties within the Lake Champlain Basin. VTANR investigators surveyed four of the sites reported to have malformations; malformed frogs were found at all four sites. Of 290 *R. pipiens* frogs examined, the incidence of malformations averaged 13.1%, ranging from 5 to 23%. The malformations were primarily missing and partial hind legs.

In late July 1997, VTANR investigators, with help from the U.S. EPA, the U.S. Fish and Wildlife Service, the U.S. Geological Survey, and Middlebury College in Middlebury, Vermont, surveyed > 50 sites in Vermont in an effort to document the extent and prevalence of malformations. Adequate sample sizes (> 50 frogs) were found at 19 of the sites surveyed. *R. pipiens* was targeted; 1,475 metamorphs were collected and examined. Roughly 8.0% of the frogs had malformations; the rates ranged from 2.0 to 45.4%. Malformations were primarily missing/partial limbs and shortened/missing digits. Fifty-seven percent of the malformations were missing or partial hind limbs, followed by 11.2% with shortened hind digits. Malformed frogs from five of the sites were sent to the National Wildlife Health Center laboratory for characterization of external and internal malformations, parasites, and viral and bacterial diseases.

In September 1997, investigators from the VTANR and the collaborating institutions resurveyed 15 of the 19 sites surveyed in July 1997. The overall malformation rate was similar to the July survey. Of 1,063 *R. pipiens* metamorphs examined, 7.3% had malformations. However, the rates of malformations observed at several sites varied significantly higher or lower as compared to the July results. The categories of malformations were similar to those in the July findings: primarily missing/partial limbs and shortened/missing digits. In 1997, Vermont citizens and volunteers reported malformed frogs from 53 towns representing all 14 counties. Although these reports are valuable, most are not verified and may not accurately represent the incidence of malformed frogs above the normal background level of ~ 1.0% because of the small sample size.

The widespread reports of malformed frogs in Vermont have made it difficult to link a particular land use with reported malformed frog sites. Chemical characterization of water and sediment from select control and affected sites is currently underway. Water and sediment from these sites is also being screened using FETAX.

Environmental Chemistry and Hydrogeology

The role of transformation and transport of potential toxicants in the aquatic environmental is an important issue that is often neglected in discussions and in experimental hypotheses to model environmental health effects. Increasingly, we are aware that environmental degradation of man-made compounds can lead to products with different characteristics which are occasionally more potent than the parent compound. We may need to consider man-made agents more within a context of the variation of their effects depending on the water matrix (alkalinity, hardness, conductivity, pH, etc.), and based on the naturally bioactive components that may be in the environment, such as phytoestrogens or autoregulators produced by microorganisms. The environmental chemistry and hydrogeology of areas where malformed frogs have been observed are likely to have important roles if causative agents are transported in the water.

Hydrogeology of selected Minnesota wetlands associated with abnormal frogs. In Minnesota, many wetlands are located in the north–central hardwoods forest (NCHF) ecoregion. This area was typically dominated by glacial advances and retreats, which produced a hummocky rolling terrain. Wetlands in this ecoregion can be completely surrounded by woods, although prairie grasses and agriculture are often mixed in the landscape. The content of soils and native vegetation vary as a function of material deposited by the most recent glacial activity and the local drainage characteristics. Landscape terrain, vegetation, soils, and the underlying stratigraphy influence the recharge and movement of groundwater in and out of the NCHF region wetlands. The current list of frog study wetlands shows many differences, including size, vegetation diversity, underlying sediments (biogeochemistry, morphology, and watershed), and variable pathways of inflow and outflow.

Assessments of the areas involved analysis of topographic maps, the measure of hydraulic heads, where possible, and the sampling of domestic water wells near the frog study wetlands. Domestic water wells were sampled for major cations, anions, stable isotopes of hydrogen and oxygen, chlorofluorocarbons (CFCs), helium, tritium to characterize flow, and hydraulic residence times. Isotopic and CFC data are currently unavailable. Many of the frog study wetlands are located midway between upgradient terrestrial recharge areas and downgradient lakes and/or rivers. Limited hydraulic head data suggest that some of the wetlands are likely flowthrough systems. Preliminary geochemical data indicate that many of the domestic water wells contain reduced water with varying ionic strengths.

Transport and fate of contaminants in aquatic environments. A myriad of anthropogenic compounds are continuously released into the environment, augmenting those already present. Contaminants appear and exist as mixtures, rather than as discrete compounds, which complicates the study of their fate and effects. In the environment the composition of these mixtures may be modified by a host of physical, chemical, and biologic factors. Modification of their composition may take two forms: a sorting of the components between environmental compartments and chemical alteration of individual compounds.

Contaminant assorting is due to partitioning between the different phases present in the environment. Initially, a water-soluble fraction, dominated by the more polar components, may be formed. Loss of the more volatile species also occurs into the air. Further sorting occurs by sorption onto and off of particulate surfaces. Thus, sediments may serve as both sinks and sources of contaminants. Organics preferentially associate with organic phases present. The need to consider the actual composition of the organic phases themselves has recently become evident. Composition and porosity of the surface and duration of contact with the contaminant are critical as well. In addition, organic and inorganic contaminants associate with dissolved organics such as humics. Discrete organic phases must also be considered, for example, surface microlayers. Lipophilic pollutants may concentrate here, impacting sensitive life stages frequenting this microhabitat. The association of contaminants with the different phases influences compound bioavailability and hence toxicity. The uptake of nonpolar contaminants by organisms has also been modeled as a partitioning process between water and organismal lipids. Metals may be actively bioaccumulated. Subsequent transfer by predator/prey associations then becomes an additional transport route of concern.

Chemical alteration of individual compounds themselves is also important, significantly increasing the number of chemical species present and modifying their properties including their toxicities. Some contaminants, such as organophosphate pesticides, are subject to hydrolysis. Others may be vulnerable to microbial degradation. This may result in the complete breakdown or the production of intermediates with lesser [e.g., polycyclic aromatic hydrocarbons (PAHs) to carboxylic acids] or greater (e.g., ethoxylate detergents to nonylphenols) toxicologic relevance. Similarly, biotransformation of contaminants within higher organisms may alter toxicity. Photomodification by UV light significantly increases the toxicity of some contaminants, for example, PAHs (*15*).

The analysis of surface and groundwater samples from Minnesota provides examples of some of the above considerations. Samples that cause deformities and mortalities in indigenous and laboratory-exposed frogs were compared to those resulting in no significant effects. Extracts were complex. Organic compounds unique to the samples causing the effects of concern were detected. No single compound was found in all affected water samples. Several compounds were tentatively identified as alachlor breakdown products, although no parent alachlor was detectable.

Surface/groundwater interface and case studies. Numerous field and modeling studies have shown that groundwater supplies considerable quantities of solutes and water to surface bodies of water. In many watersheds, groundwater supplies from 50 to 90% of the surface water; direct precipitation supplies the remainder of the water. Water is removed from watersheds by evaporation, transpiration, surface runoff, and groundwater recharge. The first two processes can concentrate solutes in the surface water.

Much of the groundwater that enters surface waters does so near the shoreline. Depending on mixing conditions, this water may or may not mix quickly with the water already present. In some settings, such as wetlands and swamps, it can remain unmixed for days or months. Hydrogeologic settings are highly variable, but in general, the inflow does not enter via discrete channels (springs) but is somewhat more distributed and spread out along shorelines.

In recent years there has been growing interest in the transport of organics from groundwater to surface water. There has also been a push to document the natural attenuation (also called self- or intrinsic-remediation) of contaminants as they pass through the highly biotic sediments of streambeds and wetlands. Although tentative, site-specific, and unpublished, the early results of these studies indicate that biodegradation of chlorinated organics can be 10–100 times more rapid in the final 50 cm of groundwater flow through streambed/wetland bottoms than in the underlying aquifers that supply water to the wetland. Degradation is rapid in the bottoms, probably because bottom sediments are physiologically rich (supplied both chemically, with both surface water and groundwater solutes, and physically, with flow directions that can change temporally) and they can have sharp changes in redox conditions within small spatial scales. Therefore, conditions for degradation of organic solutes can change from slightly aerobic in the underlying aquifer to sulfate reducing to methanogenic within a few tens of centimeters. Furthermore, the hydrodynamics of surface and groundwater can result in the mixing of the two waters with a resulting physiologic richness that rarely exists in the subsurface. Substantial reductive dehalogenation of organics can occur in this interface. However, there are less than a handful of studies on this to date, and the overall effectiveness of so-called intrinsic remediation at the groundwater/surface-water interface is unproven.

Environmental and Human Health Assessments

It is likely that the issues which have risen for discussion as a result of abnormalities in natural frog populations will recur in the future from observations in other species and geographic areas. A useful integration of environmental and human health assessments from detrimental effects in any class of sentinel species requires a balanced approach that can be continuously improved by mechanistic data. Although the biologic issues are complex, a particular end point in a natural species may indicate the direction for new laboratory investigation. Similarly, basic laboratory research combined with good diagnostics may be useful in establishing what causal factors could be involved. Assessments of implications from data in a sentinel species can move in the directions of either ecologic/environmental or human health. There are important considerations and strategies for both directions that involve issues such as biologic indicators, population assessments, cross-species prediction, and new problems such as public information and alarm.

Implications of malformed frogs for ecologic assessments. The stresses identified as possible causes of frog malformation may be acting singly, or in combination, at any of the sites where malformation has been detected. The implications for ecosystem and human health associated with each stress, and the possible mechanisms for reducing their impact, are widely divergent. These disconcerting characteristics of the malformation phenomenon present two considerable challenges. First, they provide no clear guidance, either in the selection of an appropriate level of biologic organization for research or in determination of the most appropriate temporal and spatial scales of investigation. Second, the characteristics create uncertainty concerning the relative priority to be placed on human health issues or on the health and quality of affected habitats. There are several ecologic implications of frog malformation on the possible consequences of anthropogenic or natural stresses for amphibian populations and ecosystem integrity.

The life histories of amphibians may provide a uniquely wide range of opportunities for exposure to natural or anthropogenic stresses. The characteristics of these life histories (late breeding, dependence on habitat quality in water and on land, the importance of dispersal, and colonization in local population persistence) may, in addition, predispose amphibian populations to be sensitive to perturbations of the environment. Although it is important to explore the implications of a variety of possible influences on amphibian survival and reproductive rates, ecologists are constrained by a lack of high-quality quantitative analyses of amphibian population processes. In particular, priority should be placed on obtaining a more detailed understanding of minimum viable population sizes, the influences of behavioral and competitive factors on population sizes, and more reliable data concerning population growth and replacement rates (*32*). Without these data, it will not be possible to generate predictions of population persistence or to determine how damaging different levels of malformation may be to amphibian populations in the long term.

Certain ecotoxicologic procedures may help determine whether or not a xenobiotic agent is responsible for a significant proportion of the deformities detected in Minnesota. Detoxifying enzymes may be induced through exposure to certain inorganic and organic pollutants, and a variety of assays may be performed on amphibian tissues, or tissues of other organisms, to seek evidence of exposure to pollutants. Knowledge of specific inductions may assist in the identification of the agent responsible, target chemical analysis and bioassay procedures, and provide biomarkers for rapid evaluation of the distribution and extent of contamination. For example, a broad spectrum of pesticides are inducers of mixed-function oxidases, including cytochrome P450.

Bioassays of contaminated water with a range of pond and wetland organisms may also determine critical concentrations of pollutants that present a hazard to the broader community of organisms that inhabit contaminated sites. If a xenobiotic is identified as a causal agent, enhanced regulatory protection of sites would be based on a knowledge of hazardous concentrations (*33*). A broader knowledge of the risks posed to communities of aquatic organisms would also provide initial insight into the possible inhibitory effects of pollution on ecosystem processes.

Application of laboratory* Xenopus laevis *(FETAX) data to ecologic and human health issues. The 4-day embryolarval developmental toxicity assay FETAX has been used as a surrogate to monitor various sites around the world for potential ecologic hazards to both amphibians and higher organisms. Surrogate developmental toxicity models such as FETAX are designed to simulate, but not necessarily replicate, a traditional test system. The primary objective is to provide information on larger scale issues such as indigenous species response, ecologic and ecosystem responses, human health concerns, and prioritization of future research efforts. Common considerations for surrogate models include phylogenetic relationship, relevance, reliability, time and cost effectiveness, and versatility.

Extensive testing has been conducted with FETAX in a variety of applications. The *Xenopus* model has been used to screen for developmental toxicants alone and in environmental mixtures; to study mechanisms of action and modes of biotransformation; to evaluate the effects of chemicals and environmental mixtures on limb development, thyroid function, and reproduction; as a molecular model; and as a model for evaluating nutritional essentiality.

Abnormalities induced in *Xenopus* by water and sediment samples collected from selected sites in Minnesota corresponded with field studies: malformed larvae induced by samples from ponds where abnormal frogs were identified and different types of abnormalities were induced by different water sources. Water from several sites where frogs had abnormal limb development induced hind limb deformities in *Xenopus* and significant developmental delay. As a consequence of these results, studies are currently being performed to identify probable causative agents and to evaluate the influences of physical/chemical matrices and ionic imbalance on toxicity of waterborne agents. Early in the studies, samples from selected ponds throughout Minnesota showed that the water matrix was low in ionic strength and was imbalanced. Further study of the influence of the ion matrix on *Xenopus* development using laboratory prepared simulated pond waters concluded that the Minnesota-like ionic imbalance did not directly induce the abnormalities. However, it appears that the water matrix and natural agents may act to enhance or ameliorate the toxicity of the water and should not be overlooked as a factor when considering suspected toxicants in the aquatic environment. The investigation is now focused on several organic compounds identified in affected sites. In one case there may be some contribution to the FETAX results by levels of nickel in the water.

One of the most significant contributions provided by FETAX testing thus far has been to help prioritize efforts and to identify possible toxic effects that may be associated with natural and man-made agents in the environment. Although positive results have been obtained with FETAX for some water in some wells, the initial response is to view the information conservatively with respect to any actual human health risk until the factors in the samples that caused the positive response are precisely identified and evaluated. Within the context of a relationship between sentinel species responses and potential environmental or human health effects this should be viewed as an opportunity. An understanding of the factors that may cause errors is essential and, in the public health arena, a false positive may be more desirable than a false negative.

The Centers for Disease Control and Prevention (CDC) perspective on the relevance of the reports of frog malformations to human malformations. CDC investigators do not know if the reported frog malformations are relevant to human malformation. It is not known what agents are causing the frog malformations, much less if these agents pose a risk to human development. The data from the frog studies to date are insufficient to warrant an epidemiologic study of human birth defects in states where there are high rates of malformed frogs. If in the future there are sufficient data to warrant an epidemiologic study, the CDC is prepared to assist state health departments in carrying out investigations if asked to do so.

Several types of epidemiologic studies could be conducted if it appears that human populations might be exposed to environmental agents that could cause birth defects: cluster investigations, case–control studies, or cohort studies. These studies can be difficult to conduct because human birth defects are uncommon occurrences. A major malformation is present in approximately 1 in every 30 babies born in the United States. Even the most common specific birth defects occur only about once in every 1,000 births. Most specific birth defects occur only once in every 10,000 to 100,000 births. To detect a moderate increase in the relative risks of specific birth defects in a case–control study, hundreds of cases and controls might be required. In a cohort study, thousands or tens of thousands of exposed and unexposed pregnancies might be required to detect a moderate increase in the relative risk of a specific birth defect.

Before we conduct epidemiologic studies to assess human risks of birth defects, we need more definitive information about the agents that are causing the frog malformations. More information is also needed on the types of malformations produced in frogs and their prevalence. Good data are needed on exposure and specific outcomes of exposure from environmental and toxicologic studies to help epidemiologists formulate an appropriate case definition and select appropriate exposures to investigate. In addition, it would be helpful to use birth defect surveillance systems in place in the states to be studied. State-based birth defect surveillance systems can provide baseline rates of specific malformations that might be studied, can monitor for high rates of specific defects in an area that may warrant follow-up studies, and can provide a registry of cases for follow-up studies. The data available from an ongoing surveillance system may permit epidemiologic studies to be conducted that otherwise might not be feasible. At present, 24 states have birth defect surveillance systems that provide good to adequate data on major birth defects. Several other states are presently planning or implementing such systems. The CDC encourages the development of such systems and hopes that in the near future all 50 states will have birth defect surveillance programs in place.

An EPA perspective on water quality monitoring strategies. The U.S. EPA Office of Science and Technology has an ongoing process to develop chemical-specific criteria for the protection of aquatic life. In the event a particular chemical or stressor is identified as contributing to the amphibian malformations, the development of a water quality criterion (WQC) may be necessary.

A WQC can be thought of as the highest concentration of a pollutant in water that does not represent an appreciable risk of adverse effect to aquatic organisms or human health. The criterion places limits on a particular pollutant or a condition to protect and support the designated use of the body of water. Traditionally, criteria have represented a chemical measure; presently, criteria represent a chemical, physical, biologic, or microbiologic measure that protects a body of water for a specific use.

The development of an aquatic life criterion for a specific chemical is based on aquatic toxicity data from eight families of organisms, one of which is a chordate (i.e., amphibian). If toxicity data are available on amphibians they can be used in deriving the criterion; however, there are limited amphibian toxicity data available. Comparatively, biologic criteria are developed based on the basic biology of the system rather than on toxicity information. An index of biologic integrity is identified based on the review of a gradient of impacted sites. Thus, a biologic criteria for amphibians would identify the types and distribution of species, type of habitat, and basic community composition necessary to maintain an amphibian population. This framework provides a greater integrated pathway assessment of the factors and ecosystem necessary to support amphibians. The EPA will continue to utilize these criteria and any other available methods for the protection and survival of amphibian populations.

A Minnesota public health perspective. Much of the national and international interest generated by the discovery of malformed frogs has focused on whether these animals are an indicator of environmental and public health problems. Research in progress will provide information that will certainly be of value in determining the cause or causes for Minnesota's deformed frogs. However, from a public health perspective, research reported to date has provided little or no information that would be of use in determining whether there are human health implications in the malformed frog issue. The agent, or more likely agents, responsible for the frog deformities are still unknown.

Several steps are necessary to establish a link between laboratory and field tests and possible human effects. The first is to determine if the causative factor is indeed a human health hazard. Frogs may be sentinels for the environment, but something that can cause a deformity in frogs does not necessarily mean that it has a similar effect in people. Parasites, UVB radiation, and chemicals in the water have all been suggested as causative factors. If parasites and/or UVB radiation are responsible for the observed developmental problems in frogs, there would be a reduced potential for human health problems. If the deformities are the result of the exposure of frogs or their eggs to a chemical(s) in the water, a potential for human health effects may be present—assuming that the chemicals(s) or compounds(s) are not uniquely toxic to frogs. The chemical composition of the water must be determined before any judgments about human health hazards can be made. If a hazard can be identified, the next steps would be to determine whether there is a potential for human exposure and if the material was occurring in a concentration high enough to cause an effect. The most likely route for significant exposure for humans is through the use of the water as a source for drinking water. Therefore, the potential for movement of materials from pond water to groundwater and drinking water would be a critical concern. The Minnesota Department of Health [(MDH); St. Paul, Minnesota], at the request of the MPCA, used a standard drinking water screen to test well water samples from four MPCA sites where deformed frogs had been found and to test water that was positive in the FETAX assay. This screen involves testing for > 100 compounds including a variety of inorganic chemicals, for example, metals, volatile organics, pesticides, and other synthetic organics. None of the chemicals evaluated exceeded safe drinking water standards.

Another major issue facing agencies is how to communicate the proper level of concern without causing undue public alarm. Health departments in general are very aware that public alarm can lead to overreactions and calls for expensive human health studies that generally do not satisfy either the public or the agencies. One tool that would be of particular value in providing data to respond to issues such as deformed frogs is a birth defects information system. Such systems are necessary to provide accurate and reliable data that can be used to assess relationships between exposure to chemicals and disease occurrence. The MDH is currently attempting to create a birth defects information system.

As each of the agencies continues with its own strategies for addressing the deformed frog issue, it will be critically important for all agencies involved to maintain open lines of communication. This will help to foster an atmosphere of cooperation that not only makes sense scientifically, but will also ensure that public health concerns are addressed as rapidly as possible.

Conclusions

The reported increased incidence of malformations in natural frog populations is another example of an environmental sentinel providing a warning of environmental deterioration with possible significance for human health. It is not yet clear how or if this observation is related to the general decline in amphibian species. The graphic nature of the malformations and their association with what were thought to be clean bodies of water has, understandably, led to a high level of public interest and concern. As a consequence of that widespread concern, we have an immediate responsibility to give deliberated consideration to developing integrated studies for assessing the implications for environmental and human health.

The Strategies for Assessing the Implications of Malformed Frogs for Environmental Health workshop at the NIEHS provided a forum for presentation and discussion of factors that might contribute to the induction of malformations and of the types and sequence of investigations that would most efficiently lead to an understanding of causal factors and their implications for environmental and human health. Participants in the workshop included representatives of state and federal health and environmental agencies as well as academic scientists and the public.

The primary consideration should be given to the indicating species, in this case frogs. This requires the direct supported involvement of experienced field scientists to evaluate the nature and extent of the detrimental effects as compared to what is known about the historical incidence of such effects. A well designed survey of environmental populations would be a major contribution to this task. Subsequently, a thorough diagnostic evaluation of malformed frogs is essential to provide information about causal factors and biologic systems that may be disrupted or altered. From this information, hypotheses can be proposed and tested in both laboratory models and field studies. Ultimately, the scientific process needs to identify potential causal factors, provide clear evidence of association between exposure to these factors and the resulting adverse effects, generate data that permit estimation of the relationship between exposure and adverse effects, and, finally, assess risks to both human and environmental health. If we assume that the basis for these effects is factors in the aquatic environment, an understanding of the origin, fate, and transport of the causal factors becomes an important component of the risk assessment process.

The investigation of deformities in frogs has led to basic questions regarding the best scientific approach to understanding adverse environmental effects on wildlife species and determining the implications of such effects for human health. Solving such problems will require cooperation between state and federal agencies and collaboration among chemists, toxicologists, field and research biologists, and hydrogeologists.

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